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14. ABSTRACT

Concept: We propose to investigate MYB as a potential molecular target of telomestatin in CML, and we will seek to answer two major questions about telomestatin in CML. How does telomestatin prevent MYB expression in CML, and is telomestatin active against CML in vivo? We hypothesize that the anti-leukemia activity of telomestatin in CML is in part due to inhibition of MYB expression by the binding of telomestatin to a G-quadruplex in the MYB promoter. Aims: The specific objectives of this proposal are: 1) to evaluate the ability and mechanism of telomestatin to selectively suppress c-MYB expression in K562 and K562R CML cells in culture; and 2) to investigate the anti-leukemia activity of telomestatin in murine K562 and K562R xenografts. In aim 1, we will treat imatinib sensitive and resistant CML cells with telomestatin alone or in combination with imatinib and measure the expression of C-MYB, housekeeping control genes, a panel of other genes that we have identified as containing potential G-quadruplex forming units in their promoters, and telomerase (hTERT) expression. We will show that telomestatin can prevent transcription factor binding to the MYB promoter in solution by EMSAs and footprinting, and in CML cells by ChIP assays. In aim 2, we will form K562 and K562R xenografts in immunodeficient (scid) mice, administer telomestatin to these mice by tail vein injection, and measure the activity of telomestatin on the growth of these CML xenografts. To determine the in vivo mechanism of action of telomestatin, tumors will be harvested and analyzed by standard histopathology, immunohistochemistry for MYB, and analysis of telomerase activity.

15. SUBJECT TERMS
GUANINE QUADRUPLEX, C-MYB, TRANSCRIPTION, PROMOTER, TRANSCRIPTION FACTORS, TELOMESTATIN, MURINE XENOGRAFTS, TELOMERASE, TELOMERE

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Introduction

Telomestatin is a natural product that can bind to G-quadruplex DNA, and telomestatin was reported to possess anti-leukemia activity in CML cells. The project funded by the CMLRP was to explore the activity and potential mechanism(s) of action of telomestatin in CML. Because we had recently discovered a region of the c-myb capable of forming a G-quadruplex, and because c-myb plays an important role in the malignant phenotype of CML, we hypothesized that the anti-leukemia activity of telomestatin in CML is in part due to inhibition of MYB expression by the binding of telomestatin to a G-quadruplex in the MYB promoter.

The specific objectives of this proposal, as written in the original proposal, were:

1. to evaluate the ability and mechanism of telomestatin to selectively suppress c-MYB expression in K562 and K562R CML cells in culture;
2. to investigate the anti-leukemia activity of telomestatin in murine K562 and K562R xenografts.

In aim 1, we proposed to treat K562 with telomestatin, using telomestatin that was provided to a co-investigator, Dr. Hurley, by Dr. Shin-Ya from the University of Tokyo. We proposed to measure cell proliferation by MTS assay to determine the IC50, and then measure the expression of MYB compared to housekeeping control genes (β-actin and GAPDH) by real time PCR. We proposed to evaluate selectivity for MYB against a panel of other cancer-related genes identified in the Hurley and Ebbinghaus laboratories as containing potential G-quadruplex forming units in their promoters. These studies would determine whether and how telomestatin prevents MYB expression in CML cells, and we reasoned that even if these studies did not demonstrate MYB inhibition by telomestatin, useful information on the mechanism and activity of telomestatin in CML would be gained. Based on the work *in vitro,* we proposed to evaluate the anti-leukemia activity of telomestatin in a *scid* mouse models of CML.

Body

MYB amplification or overexpression in leukemias is an important part of the molecular signature of CML. MYB is differentially overexpressed by more than 6-fold in CML in chronic phase (CML-CP) and more than 10-fold in CML blast crisis (CML-BC). Functions of potential significance to hematopoietic cell transformation might relate to MYB's ability to regulate hematopoietic cell proliferation, MYB's effect on important cell cycle genes like c-MYC, or MYB's role in regulating hematopoietic cell differentiation.

A polypurine tract (PPT) containing multiple GGA repeats is an important promoter element in the control of c-myb transcription. GGA repeats have been shown to form unusual DNA structures related to guanine (G) quadruplexes at physiological potassium concentrations. G quadruplexes are emerging as potential therapeutic targets for the treatment of cancer. We had preliminary evidence that the PPT might be able to fold into an intramolecular quadruplex within cells, specifically a structure called a tetrad:heptad (**Figure 1**), composed of a typical guanine tetrad stacked on a guanine-adenine heptad

containing 4 guanines and 3 adenines. Quadruplex formation in the c-myb promoter could be stabilized by the addition of DNA interactive compounds, such as telomestatin, to suppress c-myb transcription.

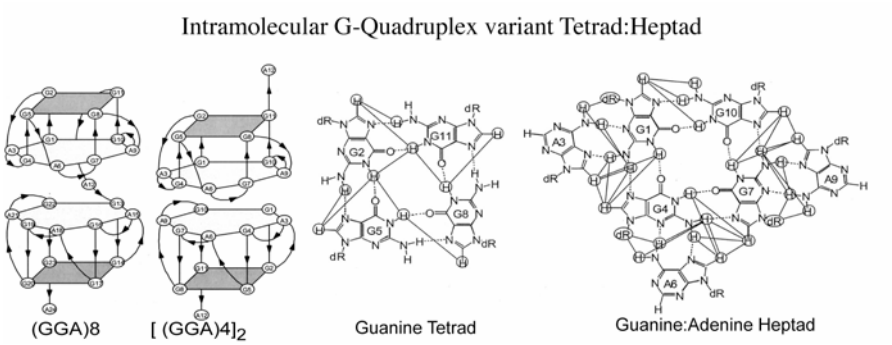

Figure 1. Tetrad:heptad DNA structures formed by oligos with four or eight GGA repeats.

Charaterization of Secondary Structure Formation by the c-myb GGA Repeats

Our studies of G-quadruplex formation by the c-myb promoter present some of the most compelling evidence to date for the relevance of G-quadruplex structures in the control of gene expression in living cells. Our first manuscript resulting from this work was submitted to *Nucleic Acids Research* on May 31, 2007. In brief, chemical footprinting, circular dichroism, and RNA and DNA polymerase arrest assays on oligonucleotides representing the GGA repeat region of the c-myb promoter demonstrate that the element is able to form tetrad:heptad:heptad:tetrad (T:H:H:T) G-quadruplex structures by stacking two tetrad:heptad G-quadruplexes formed by two of the three $(GGA)_4$ repeats. Deletion of one or two $(GGA)_4$ motifs increases c-myb promoter activity, and the relative promoter activity is inversely correlated with the stability of the G-quadruplex formed in the remaining $(GGA)_4$ motifs. Complete deletion of the c-myb GGA repeat region abolishes c-myb promoter activity, indicating dual roles of the c-myb GGA repeat element as both a transcriptional repressor and an activator. Furthermore, we demonstrated that Myc Associated Zinc finger (MAZ) represses c-myb promoter activity and binds to the c-myb T:H:H:T G-quadruplexes. Our findings show that the T:H:H:T G-quadruplex forming region in the c-myb promoter is a critical *cis* acting element and may repress c-myb promoter activity through MAZ interaction with G-quadruplexes in the c-myb promoter. Please refer to the attached manuscript for the experimental details and a full discussion of the results obtained for this important paper.

Evaluation of Telomestatin on MYB Expression in K-562 Cells

We evaluated the ability of telomestatin to suppress c-myb expression in K-562 cells. Cells were treated with increasing concentrations of telomestatin over a range of times, as shown in Figure 2. c-myb mRNA was measured by real time PCR, and normalized with B-actin and GAPDH. We found no change on c-myb expression due to telomestatin treatment at up to 96 hours, as shown in Figure 2, and we only observed a 40% decrease

in c-myb mRNA levels after 7 days exposure to 10 uM telomestatin, conditions which appeared generally toxic to the cells.

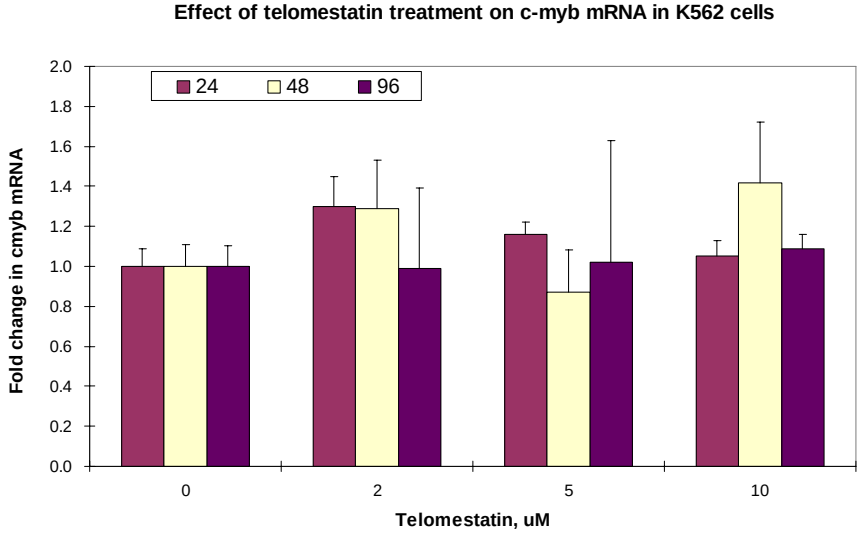

Figure 2. Telomestatin treatment of K-562 cells did not reduce c-myb expression.

From these data, reproduced in multiple experiments, we concluded that c-myb was probably not a molecular target for telomestatin in its anti-leukemic effects on K-562 cells.

The discovery of a G-quadruplex forming region of the hTERT promoter.
Telomestatin was previously reported to induce telomere shortening and inhibit telomerase activity in TRAP assays (Tauchi T. et.al., *Oncogene.* 2003 Aug 14;22(34):5338-47). To verify that we were using a "bioactive" preparation of telomestatin, we performed TRAP assays with K-562 extracts treated with telomestatin, and our results were generally in agreement with the published data, showing >80% inhibition of telomerase activity. However, it is important to note that the TRAP assay does NOT truly evaluate the effect of a compound on the endogenous telomere of the treated cell; rather, in this context, it is a bioassay for the level of functional telomer*ase* enzyme in the cell extracts being tested. Furthermore, in the Tauchi paper describing the effects of telomestatin in CML cells, the authors reported a decrease in hTERT mRNA levels—an observation that we also reproduced. From these studies, we were motivated to re-examine the concept of the telomere as the molecular target of telomestatin. Rather, we hypothesized that the data from these studies in our lab and previously published studies can be re-interpreted to indicate that hTERT, the gene producing the telomerase enzyme, is the molecular target of telomestatin.

We examined the hTERT promoter and found that the critical cis-acting region that contain or overlap with essential Sp1 binding sites contained runs of guanines in motifs that were potentially capable of forming one or more intramolecular guanine quadruplexes (Figure 3). Using synthetic oligomers, we used footprinting and circular

dichroism to show that the hTERT promoter contains sequences capable of G-quadruplex formation. These data lead us to the hypothesis that G-quadruplex formation in the promoter region of the hTERT gene

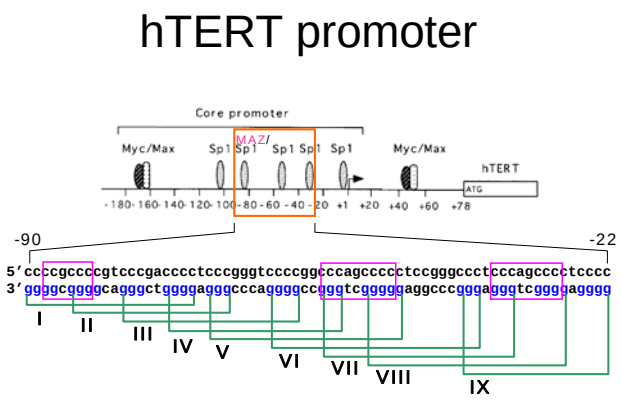

Figure 3. Potential G-quadruplex forming sequences in the hTERT promoter are found in the critical cis-acting element of the core hTERT promoter.

We used a cell-free drug-screening assay, the DNA polymerase arrest assay, to show that telomestatin, as well as the cationic porphyrin TmPyP4, but not its positional isomer, TmPyP2, could interact with the G-quadruplex formed by the hTERT promoter.

The biological significance of these observations is emerging. It is clear that G-quadruplex interactive compounds can decrease hTERT expression by leading a decrease in the hTERT mRNA levels. In our studies, we confirm the observations of Tauchi, et al, by showing that telomestatin can decrease hTERT mRNA levels, and we have extended these observations to include the cationic porphyrin G-quadruplex binding agents. It is also notable that a decrease in hTERT mRNA levels was also reported after treatment with the G-quadruplex interactive compound 12459 by Gomez, et al (*Nucleic Acids Res*. 2004 Jan 16;32(1):371-9), and shown in this paper to result from alternate mRNA splicing. Regardless of mechanism, three independent groups (ours and two others), using different G-quadruplex binding compounds and different cell lines, have now shown that G-quadruplex interactive compounds can suppress hTERT mRNA levels.

To determine whether the decrease in endogenous hTERT mRNA levels is due to a decrease in hTERT transcription by an interaction of the drug with the hTERT promoter, we constructed a cell-line called "293 hTERT P330-luc," containing the 330 bp core hTERT promoter sequence driving the expression of luciferase, integrated into the genome of 293 cells in single copy by using a homologous recombination strategy. We showed that telomerase (not shown) and TmPyP4 (**Figure 4**) significantly decrease hTERT promoter activity in 293 cells

P2 and P4 treatment on 293 hTERT P330 cells

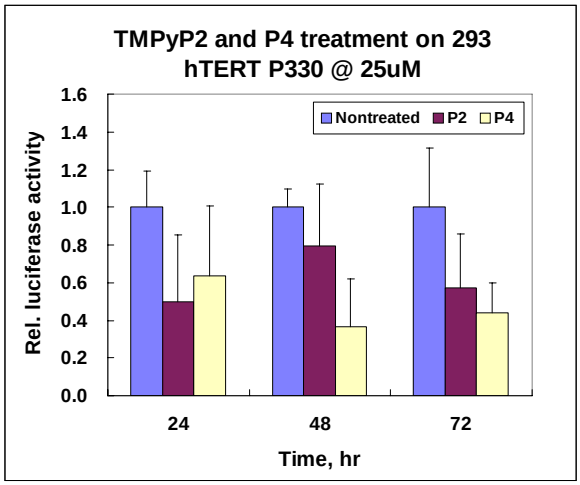

Figure 4. The cationic porphyrin and G-quadruplex binding drug, TmPyP4 leads to a significant reduction in hTERT driven luciferase activity. A smaller effect is also seen with the positional isomer, TmPyP2.

In follow-up, we have shown that hTERT expression falls in a concentration dependent manner in response to treatment with TmPyP4, and at the 100uM concentrations previously required to suppress c-myc expression, the fall in hTERT expression occurs within hours and precedes the fall in c-myc mRNA.

In our work on the c-myb promoter, we showed that the transcription factor MAZ can bind to the G-quadruplex and may be a negative regulator of c-myb expression by binding to the c-myb G-quadruplex (see attached manuscript). Since MAZ is also known to bind in the hTERT promoter in an area that overlaps with the G-quadruplex forming elements (see Figure 3), we speculated that MAZ might be involved in downregulating hTERT expression by recognition of and binding to the G-quadruplex formed in the hTERT promoter. We showed that the forced overexpression of MAZ in the 293 hTERT P330-luc cells markedly decreases hTERT expression, and we have very recently shown by EMSA that MAZ protein can bind specifically to the G-quadruplex conformation of the hTERT promoter.

In conclusion, we propose that the hTERT promoter is a molecular target for telomestatin and other G-quadruplex binding drugs. While the c-myb promoter does not appear to be a molecular target of telomestatin in the treatment of K-562 cells, we have shown that myb regulation very likely involves the formation of an unusual G-quadruplex structure, a tetrad:heptad:heptad:tetrad, and the interaction of a transcription factor, MAZ, with double-stranded and G-quadruplex conformation of the promoter.

Key Research Accomplishments

- Biochemical characterization of the G-quadruplex formed by the c-myb promoter, and submission of manuscript describing these results to *Nucleic Acids Research.*.
- Drug testing of telomestatin on K-562 cells, and analysis of target gene expression.
- Discovery of the G-quadruplex forming region of the hTERT promoter.
- Elucidation of the mechanism of action of telomestatin by demonstrating that G-quadruplex ligands suppress hTERT expression by a direct interaction with the hTERT promoter.

Reportable Outcomes (reprints, presentations, patents, etc.)

Submitted Manuscript

SunMi Lee Palumbo, Regan M. Memmott, Diana J. Uribe, Yulia Krotova-Khan, Laurence H. Hurley, and Scot W. Ebbinghaus, A novel G-quadruplex forming GGA repeat region in the c-myb promoter is a critical regulator of promoter activity, submitted to *Nucleic Acids Research* on May 31, 2007.

Conclusions

In conclusion, we propose that the hTERT promoter is a molecular target for telomestatin and other G-quadruplex binding drugs. While the c-myb promoter does not appear to be a molecular target of telomestatin in the treatment of K-562 CML cells, we have shown that myb regulation very likely involves the formation of an unusual G-quadruplex structure, a tetrad:heptad:heptad:tetrad, and the interaction of a transcription factor, MAZ, with double-stranded and G-quadruplex conformation of the promoter.

References cited

None

Appendix

Copy of manuscript submitted to *Nucleic Acids Research*

OXFORD JOURNALS
Nucleic Acids Research

A novel G-quadruplex forming GGA repeat region in the c-myb promoter is a critical regulator of promoter activity

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Manuscript ID:	NAR-01215-D-2007
Manuscript Type:	1 Standard Manuscript - US Editorial Office
Key Words:	myc associated zinc finger (MAZ), leukemia

Running Title: A novel G-quadruplex forming GGA repeat region in the c-myb promoter is a critical regulator of promoter activity

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ABSTRACT

The c-myb promoter contains multiple GGA repeats beginning 17bp downstream of the transcription initiation site. GGA repeats have been previously shown to form unusual DNA structures in solution. Results from chemical footprinting, circular dichroism, and RNA and DNA polymerase arrest assays on oligonucleotides representing the GGA repeat region of the c-myb promoter demonstrate that the element is able to form tetrad:heptad:heptad:tetrad (T:H:H:T) G-quadruplex structures by stacking two tetrad:heptad G-quadruplexes formed by two of the three $(GGA)_4$ repeats. Deletion of one or two $(GGA)_4$ motifs increases c-myb promoter activity, and the relative promoter activity is inversely correlated with the stability of the G-quadruplex formed in the remaining $(GGA)_4$ motifs. Complete deletion of the c-myb GGA repeat region abolishes c-myb promoter activity, indicating dual roles of the c-myb GGA repeat element as both a transcriptional repressor and an activator. Furthermore, we demonstrated that Myc Associated Zinc finger (MAZ) represses c-myb promoter activity and binds to the c-myb T:H:H:T G-quadruplexes. Our findings show that the T:H:H:T G-quadruplex forming region in the c-myb promoter is a critical *cis* acting element and may repress c-myb promoter activity through MAZ interaction with G-quadruplexes in the c-myb promoter.

INTRODUCTION

The c-myb proto-oncogene, the cellular homologue of the transforming v-myb oncogene of avian leukemia viruses, encodes a critical transcription factor for proliferation, differentiation, and survival of hematopoietic progenitor cells (1). High levels of the gene product, c-Myb, prevent hematopoietic stem cells from both differentiation and apoptosis (2;3). Because of the critical role of c-Myb in determining cell fate, c-Myb expression levels are tightly controlled in normal cells, showing high levels in immature, proliferating hematopoietic cells and undetectable levels in differentiated cells (4). c-Myb is overexpressed in many leukemias and some solid tumors, and plays a critical role in leukemogenesis by maintaining cells in a proliferative state and by preventing terminal differentiation (5-7). Luger and colleagues showed that an antisense oligonucleotide against c-myb mRNA could eliminate leukemia cells as a bone marrow purging agent, resulting in cytogenetic remissions in CML and showing that c-myb is a potential therapeutic target for leukemia treatment (8).

Cellular levels of c-Myb are regulated at the transcriptional level by several mechanisms. One important mechanism is blocking mRNA elongation in intron I, which attenuates c-myb mRNA elongation in a cell type dependent manner (9-12). c-Myb transcription is also controlled by a number of transcription factors. For example, c-Myb acts as a negative regulator of its own expression in a lineage dependent manner (13). WT1, MZF1 (myeloid zinc finger 1), and PU.1 also downregulate c-myb promoter activity, and the Ets and c-Jun/JunD transcription factors activate the c-myb promoter in a lineage specific manner (Figure 1A) (14-18).

The c-myb promoter contains a purine-rich region with three copies of four GGA repeats, [3$(GGA)_4$], located 17 bps downstream of the transcription initiation site on the bottom strand, and the GGA repeat region is highly conserved in the human and murine c-myb promoters. Nuclear magnetic resonance (NMR) and molecular modeling studies showed that $(GGA)_4$ DNA sequences similar to those found in the human c-myb promoter can form an unusual secondary DNA structure related to guanine quadruplexes and composed of a guanine tetrad (T) stacked onto a guanine-adenine heptad (H) (Figure 2B, left) (19;20). This unique G-quadruplex was the first identified G-quadruplex structure with pseudo-double chain reversal loops in which the loop base (A) is part of the heptad. DNA sequences with two adjacent $(GGA)_4$ units, $(GGA)_8$, can form a very stable (Tm = 86°C) higher ordered structure by intramolecularly stacking two T:H G-quadruplexes on the heptad plane, resulting in a

tetrad:heptad:heptad:tetrad (T:H:H:T) DNA structure (Figure 2B, right) (19;20). These structures form in the presence of physiologic concentrations of potassium ions and at neutral pH.

GGA repeats found in some promoters are shown to be a critical *cis* element for protein:DNA interaction. The eight GGA repeats $(GGA)_8$ found in the avian SPARC promoter were shown to be a critical positive regulatory region with multiple Sp1/Sp3 binding sites (21). In addition, the human homologue of SPARC, the BM-40 gene, also contains GGA repeats region and the region is required to exert promoter activity of the BM-40 gene (22). These studies show that GGA repeats in gene promoters can play critical roles in regulating gene expression, and suggest that the formation of secondary DNA structures may be important to the function of *cis*-acting GGA repeats.

In the present study we investigated if the c-myb GGA repeat region can form T:H:H:T G-quadruplexes and if the region is a critical regulator of promoter activity. We report that the c-myb GGA repeat region forms T:H:H:T G-quadruplexes involving two $(GGA)_4$ regions and acts as a repressor element of the c-myb promoter. However, the GGA repeat region is also essential for basal promoter activity of the c-myb gene. We identified a transcription factor, Myc Associated Zinc finger protein (MAZ), that binds to the double stranded and G-quadruplex conformations of the GGA repeat region and represses c-myb promoter activity. Our findings suggest that the c-myb G-quadruplexes act as a negative regulator of the promoter and that MAZ may repress c-myb promoter activity by binding to the c-myb T:H:H:T G-quadruplexes.

MATERIALS AND METHODS

Oligonucleotides

The PPG oligonucleotide ODN VII was synthesized by Bio-Synthesis, Inc. (Lewisville, TX) and other oligonucleotides were synthesized by Operon Biotechnologies, Inc. (Huntsville, AL). All the oligonucleotides were gel purified and the concentrations were determined by using a spectrophotometer. The summary and sequences of the oligonucleotides are shown in Table 1 and Supplementary Data, respectively.

Circular dichroism spectroscopy

Oligonucleotides for circular dichroism (CD) were prepared at 5μM in 50mM Tris-HCl (pH 7.5) in the presence or absence of 140mM KCl. Samples were denatured at 95°C and slowly cooled to room temperature. CD spectra were measured by a Jasco-810 spectropolarimeter (Jasco, Easton, MD) using a quartz cell of 1-mm

optical path length, an instrument scanning speed of 100 nm/min, with a response of 1s, over a range of 200 to 350nm. A set of three scans was averaged for each sample at 25°C.

Dimethylsulfate (DMS) protection assay

Oligonucleotides were 5'-end labeled, diluted to 100nM in 40ul of 10mM TrisHCl (pH 8.0), denatured at 95°C, and slowly cooled down to 37°C in the presence or absence of 140mM KCl. 1μg of dIdC was added to each sample, and the sample was treated with 0.5% DMS as a final concentration for 5 min at RT. The reaction was quenched by adding 50ul of a stop solution (1.5M sodium acetate (pH 7.0), 1M β-mercaptoethanol, 250μg/ml yeast tRNA), and the DMS treated oligonucleotides were cleaved by 10%(v/v) piperidine, and separated on a 10% sequencing gel.

Polymerase arrest assays

DNA-polymerase arrest assays were performed as previously described (23). Briefly, a primer was annealed to the template bearing the sequence of interest, and gel purified. The asymmetric primer-template duplex was incubated in the absence or presence of 140mM KCl at 37°C overnight and primer extension was conducted with Taq polymerase (Fermentas, Hanover, MD) at 47°C for 20min.

RNA polymerase arrest assays were conducted by using the *in vitro* transcription kit, T7 MEGAshortscript from Ambion (Austin, TX) according to the manufacturer's protocol. The top sequence of T7 promoter was annealed to template oligonucleotides bearing the bottom sequence of T7 promoter followed by the c-myb GGA repeat region. *In vitro* transcription from the T7 promoter site was conducted in the presence of rNTPs and ^{32}P alpha UTP for 2hrs at 37°C. The reaction was stopped by digesting DNA templates with DNase I and the transcription products were separated on a 12% denaturing gel.

Cell lines

The acute lymphoblastic leukemia cell line CCRF-CEM and the human embryonic kidney cell line HEK 293 were purchased from ATCC (Manassas, VA). The chronic myelogenous leukemia cell line K562 was purchased from Coriell Cell Repositories (Camden, NJ). CCRF-CEM and HEK 293 cells were maintained in RPMI 1640 (Cellgro, Herndon, VA) and DMEM (Cellgro, Herndon, VA), respectively, with 10% heat-inactivated fetal bovine serum at 37°C with 5% CO_2.

Site-directed mutagenesis

The wild-type c-myb reporter plasmid pMybWT was constructed by subcloning the 900bp c-myb promoter in the plasmid LB178 into a pGL3 basic vector (Promega, Madison, WI). All the mutant c-myb reporter plasmids, except for pMybDelR2, were generated by using the Quik Change XL Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) according to the manufacturer's protocol. For pMybDelR2, mutagenic PCR amplified the reporter plasmid pMybWT excluding Region 2 GGA repeats in the promoter, and then the PCR product was ligated in order to circularize it. The c-myb GGA repeat region and adjacent flanking region in the reporter plasmids were sequenced in order to confirm that there were no unintended mutations introduced during mutagenesis.

Transient transfection and luciferase assay

CCRF-CEM cells in exponential growth phase were plated at a density of $6x10^5$ cells/well of a 24-well plate in 500μL OPTI MEM (Invitrogen, Carlsbad, CA). Lipofectamine 2000 (Invitrogen, Carlsbad, CA) was used as a transfection reagent, and transient transfection was conducted according to the manufacturer's protocol, using 10ng of the renilla luciferase reporter plasmid pRL SV40 and 1μg of the c-myb reporter plasmids. For cotransfection, HEK 293 cells were plated at 50% confluency in OPTI MEM. 1μg of pMybWT was transfected with 0 to 3μg of the FLAG-tagged-MAZ expression plasmid BRB112 and 10ng of the renilla luciferase plasmid pRL TK by using Lipofectamine 2000. Firefly and renilla luciferase activities were measured by using a dual luciferase reporter assay system (Promega, Madison, WI) 24 hours after transfection. Each transfection was done in duplicate, repeated at least three times, and the data are presented as the average relative luciferase activity compared to the wild-type c-myb promoter from 6 luciferase assays per plasmid.

Nuclear extract preparation

K562 cells were harvested, resuspended in RSB buffer (10mM NaCl, 3mM $MgCl_2$, 10mM TrisHCl, pH 7.5, 0.05% NP-40, and protease inhibitors), vortexed, incubated on ice, and spun down. The pellet was washed once with RSB buffer and resuspended in RIPA buffer (50mM TrisHCl (pH 7.5), 1% NP-40, 0.25% sodium deoxycholate, 150mM NaCl, 1mM EDTA and protease inhibitors). Then, samples were vortexed, incubated on ice, and spun down. The supernatant containing nuclear proteins was stored at -80°C.

Expression of FLAG-MAZ

FLAG-tagged-MAZ was expressed from the plasmid BRB112 in BL21 (DE3) pLysS cells (Invitrogen, Cedar Creek, TX) according to the manufacturer's protocol. Briefly, BRB112 was transformed into BL21 (DE3)

pLysS cells. Overnight culture was prepared from a single colony and diluted to 1:100 in fresh Luria-Bertani broth with 0.4% glucose. Pre- and post-induction incubations were 2hr each at 37°C, and 1mM of IPTG was used for induction of FLAG-MAZ expression. After induction, bacterial cells were harvested, and lysed, and FLAG-MAZ was purified by a FLAG affinity column according to the manufacturer's protocol (Sigma-Aldrich, St. Louis, MO). FLAG-MAZ was also synthesized by using the TnT® coupled Reticulocyte lysate systems according to the manufacture's protocol (Promega, Madison, WI). Briefly, 2µg of the plasmid BRB112 was used as a DNA template in a final volume of 50 µl, and coupled transcription/translation reaction was conducted at 30 ºC for 90min.

Electrophoretic Mobility shift assay (EMSA) and Competition EMSA

For EMSA with purified FLAG-MAZ, 1ug of purified FLAG-MAZ was incubated in a binding buffer (10mM TrisHCl (pH 7.5), 50mM NaCl, 1mM dithiothreitol (DDT), 1mM EDTA (pH 8.0), and 5% glycerol) in the presence of 0.2pmols of labeled, double stranded probes and incubated for 20min at room temperature. For EMSA with FLAG-MAZ synthesized by the TnT® coupled Reticulocyte lysate system, the previously described methods in Reference (24) was followed. However, in this study 10µl of the coupled reaction was used per sample in the presence of the labeled probes at 12.5nM. For competition EMSAs, 5µg of K562 nuclear extract was incubated with 0, 5, and 20pmols of double stranded cold competitors in the binding buffer with 1µg of dIdC for 20min at room temperature and then 0.2pmols of the labeled probes were added to the reaction and incubated for 20min at room temperature. Probe-protein complexes were separated on a 4% non-denaturing gel with 2.4% glycerol in 0.25X TBE at 4ºC.

DNase I protection assay

A 260bp GGA repeat region probe was prepared by digesting pMybWT with BanII and SfcI and labeling by Klenow filling. Approximately 18,000 cpm of the probe was incubated in a binding buffer (25mM TrisHCl (pH 8.0), 50mM KCl, 6.25mM $MgCl_2$, 0.5mM EDTA (pH 8.0), 10% glycerol, 0.5mM DTT) for 10min on ice in the presence of FLAG peptides, or FLAG-MAZ. After adding $CaCl_2$ and $MgCl_2$ to 2.5mM and 5mM as final concentrations, respectively, 0.01u of DNase I (Invitrogen, Carlsbad, CA) was added to each sample and incubated 2min at room temperature. DNase I reaction was quenched by adding a stop solution (100mM NaCl, 15mM EDTA, 0.5% SDS, 50µg/ml yeast tRNA as final concentrations). The labeled probes were recovered via phenol/chloroform extraction followed by ethanol precipitation and separated on a 6% denaturing gel.

Statistical analysis

Microsoft Excel was used to perform statistical analysis. Paired sample T test with two tails was used to determine statistical differences in relative luciferase activities of the wild-type and mutant c-myb reporter constructs, and a value of $P<0.01$ was considered significant.

RESULTS

The c-myb GGA repeat region forms T:H:H:T G-quadruplexes

Oligonucleotides with the sequence $(GGA)_4$ have been shown to form a secondary structure composed of a guanine tetrad stacked on a guanine-adenine heptad. Two tetrad:heptad G-quadruplex (T:H) can dimerize by stacking interactions on their heptad planes to form a tetrad:heptad:heptad:tetrad structure (T:H:H:T) (Figure 1B, left from reference 19, permission for this figure is pending) (19). This T:H:H:T G-quadruplex can be derived intramolecularlly from a $(GGA)_8$ oligonucleotide, and was shown to be very stable, producing a melting temperature of 86°C (20). In this structure, the two T:H G-quadruplex forming core sequences, GGAGGAGGAGG, are linked by a single adenine nucleotide that does not form any hydrogen bonds to stabilize the T:H:H:T higher order structure (Figure 1B, right from reference 20, permission for this figure is pending) (20).

Since the c-myb promoter contains three nearly perfect tandem $(GGA)_4$ repeats (Figure 1A), we speculated that each could be considered a T:H forming unit, and would form T:H:H:T structures with slightly longer linker sequences than in the previously characterized $(GGA)_8$ oligonucleotide (Figure 1B, right from reference 20, permission for this figure is pending). We performed DMS footprinting studies with an oligonucleotide containing all three GGA repeat regions (ODN I) and oligonucleotides in which each region was mutated to $(GCA)_4$ to prevent secondary structure formation (ODNs II, III, and IV) (Figure 2A). These studies show potassium dependent DMS footprints in two of the three GGA repeat regions in ODN I, and in the two unaltered GGA repeat regions of ODNs II, III, and IV (Figure 2A). This pattern of DMS protection suggests that T:H:H:T structures are formed within the c-myb GGA repeats, and that any two of the three $(GGA)_4$ repeats can form the dimer structure, implying a possible competition for dimerization among the three GGA repeat regions. Significantly, the levels of DMS protection in the mutant ODNs show that T:H:H:T stability varies in an order of ODN III > ODNs I = IV >> ODN II, suggesting that R1/R3 in ODN III and R1/R2 in ODNs I and IV form a strong T:H:H:T dimer and that R2/R3 in ODN II forms a less stable T:H:H:T. When considering the partial protection of R1 in ODN I but complete protection of R1 in ODN III, these findings imply that the competition for dimerization seems to weaken the overall stability of the c-myb T:H:H:T G-quadruplexes. Furthermore the dimers with the longest and shortest linkers show the greatest (ODN III) and weakest (ODN II) stability. An

interesting observation is that R1 and R3 forming the most stable T:H:H:T are perfectly conserved in the human and murine GGA repeat regions, suggesting that R1 and R3 may cooperate to control myb expression.

Circular dichroism (CD) has been used to deduce the relative orientation (parallel vs. antiparallel) of DNA strands in G-quadruplexes(25), and most forms of G-quadruplexes produce very characteristic CD spectra. The CD spectrum of a T:H:H:T from a $(GGA)_8$ oligonucleotide (ODN V) is shown in Figure 2B, and essentially reproduces the CD signature for the T:H:H:T structure that was reported in correlation with the NMR studies from which the T:H:H:T was identified (20). ODN VI is an oligonucleotide representing the c-myb promoter and containing GGA repeat regions 1 and 2 with the flanking sequence and intervening sequence between the GGA repeats derived from the c-myb promoter. ODN VI produces the characteristic CD signature of a T:H:H:T with a strong peak at 264nm in the presence of potassium, indicating T:H:H:T G-quadruplex formation of the oligonucleotide. In order to verify that the CD signature of ODN VI is due to G-quadruplex formation of the oligonucleotide, we tested a negative control oligonucleotide (ODN VII) that has the same sequence as in ODN VI, however, in which the G-quadruplex forming guanines of ODN VI have been substituted with 8-aza-7-deazaguanines (PPGs). PPG is a guanine analog in which the N7 and C8 atoms of guanine are interposed, which prevents the formation of N7 dependent secondary DNA structures, such as G-quadruplexes. However, PPG retains the same electron density in the ring system as guanine, and PPG substitutions do not alter the optical activity (absorption of UV light) compared to the unfolded form of the guanine oligo. As shown in Figure 2B, the negative control oligonucleotide ODN VII did not produce the CD signature of a T:H:H:T, confirming that the CD signature of ODN VI is due to T:H:H:T formation. When taken together with the other biochemical studies presented here, the CD spectra provide strong supporting evidence for the formation of a T:H:H:T structure by the GGA repeat regions of the c-myb promoter.

DNA and RNA polymerase stop assays have been used as a means to show the relative stability of DNA secondary structure formation in a DNA template strand, and have been used to deduce the location and stability of G-quadruplexes in single stranded templates and plasmid DNA (26;27). Because the GGA repeat region of c-myb occurs on the transcribed strand downstream of the transcription start site, RNA polymerase arrest could occur in the c-myb promoter if a stable G-quadruplex structure was formed. Thus, both DNA and RNA polymerase arrest sites at these sequences are potentially relevant to the disruption of enzymatic function that could be encountered in cells by G-quadruplex formation in the c-myb GGA repeat region. The results show that

DNA (Figure 3A and B) and RNA (Figure 3C) polymerase arrest sites (designated S1 and S2) are observed precisely at the beginning of the third and second GGA repeat regions. Polymerase arrest occurs in a potassium dependent manner, as expected with G-quadruplex formation. Significantly, in the full-length c-myb GGA repeat templates (ODNs XI and XXI), polymerase arrest at the first arrest site (S1) is only partial, and the second arrest site (S2) is more pronounced, consistent with the data from the DMS protection assays that the T:H:H:T formed between GGA repeat regions 1 and 2 is the most preferred, when all three regions are intact. The presence of two arrest sites indicates that T:H:H:T formation between GGA repeat regions 2 and 3 also occurs, and that the c-myb promoter can form a mixture of stable secondary structures due to the interaction between adjacent GGA repeat regions.

When one of the GGA repeat regions is mutated (ODNs XII, XIII, and XIV in Figure 3A), T:H:H:T formation occurs with the intact two GGA repeats and results in a single arrest site at either S1 or S2. This observation is comparable to the single arrest site (S) that is observed before the GGA repeats in a template containing the $(GGA)_8$ sequence (ODN XVII in Figure 3B) that was used as a positive control for the T:H:H:T structure (20), and indicates that any two $(GGA)_4$ repeats can form T:H:H:T regardless of their positions in the c-myb GGA repeat region. Of note, although any two $(GGA)_4$ motifs are able to form a T:H:H:T, the relative amount of arrest product compared to full-length (FL) primer extension products varies in ODNs XI, XII, XIII, and XIV, which provides an estimate of the stability of the T:H:H:T structures formed by the GGA repeat sequences. For example, in Figure 3A, ODN XI (representing the WT myb sequence) shows two strong arrests at S1 and S2 with no full-length primer extension product, whereas ODN XII shows only partial arrest at S1 with a substantial amount of full-length primer extension product. The percentage of arrest products in Figure 3A demonstrates an order of stability of ODN XI = ODN XIV > ODN XIII > ODNXII. These data are in agreement with the stabilities determined by the DMS footprints in Figure 2A, and both assays demonstrate that deletion of GGA repeat region 1 leads to a weak T:H:H:T structure formed between R2 and R3.

Deletion studies in DNA and RNA polymerase arrest assays demonstrated that dimerization of two T:H G-quadruplexes is required for stable quadruplex formation. When one of the GGA repeat regions is deleted (ODNs XV and XXV in Figure 3B and C), T:H:H:T formation takes place by dimerization of two T:H quadruplexes and causes a single arrest site in both DNA and RNA polymerase assays. However, when two GGA repeat regions are deleted (ODNs XVI and XXVI), a single T:H structure could form, and this structure

partially arrests RNA polymerase (Figure 3C) but not DNA polymerase assays (Figure 3B), suggesting that the T:H structure is not as stable as the T:H:H:T structure, in support of the concept that stacking interactions between the heptad planes of two T:H subunits leads to a more stable higher order T:H:H:T structure.

In summary, these biochemical assays demonstrate that two $(GGA)_4$ motifs from the c-myb promoter can form a stable potassium dependent secondary DNA structure capable of arresting the progress of DNA and RNA polymerases, and provide evidence for the formation of T:H:H:T structure by the c-myb promoter.

The c-myb GGA repeat region plays a critical role in c-myb promoter activity

In order to investigate the role of the GGA repeat region in c-myb promoter activity, we performed luciferase assays using wild-type and mutant c-myb promoters. The reporter plasmid pMybWT bears the wild-type c-myb promoter from -719 to +200 with the three intact GGA repeat regions. The mutant reporter plasmids pMybDelR1, pMybDelR2, and pMybDelR3 are deletion mutants in which a $(GGA)_4$ motif has been individually deleted as shown in Figure 4. We also generated serial deletion mutant plasmids, pMybDelR1/2 and pMybDelR1/2/3, in which two or all three GGA repeat regions have been deleted. Figure 4 shows relative firefly luciferase activities in CCRF-CEM cells normalized by renilla luciferase activities in a dual luciferase assay system. Deletion of GGA repeat regions 1, 3, or both 1 and 2 in the c-myb promoter increased relative luciferase activities by about 3 to 5-fold. However, deletion of region 2 did not show a significant increase in luciferase activity. Interestingly, the fold changes in luciferase activity of the regional deletion mutants in CCRF-CEM cells are inversely correlated with the stability of G-quadruplex formed in the mutant plasmids (Figure 4). For instance, T:H:H:Ts are able to be formed by R2/R3 in pMybDelR1, R1/R3 in pMybDelR2, and R1/R2 in pMybDelR3. Also, a T:H could be formed by R3 in pMybDelR1/2. Based upon our results from the polymerase arrest assay (Figure 3A), the stability of the T:H:H:Ts formed in the mutant constructs would be predicted in order of pMybDelR2 > pMybDelR3 > pMybDelR1 > pMybDelR1/2 and this order is inversely correlated with promoter activity of the mutants constructs (pMybDelR1 ≈ pMybDleR1/2 > pMybDelR3 > pMybDelR2). These results suggest that strong T:H:H:T formation may be important for repression of c-myb promoter activity. Broadly similar results were observed in two other leukemia cell lines, Jurkat and K562 (data not shown), but the magnitude of the effects of the deletions was dependent on the cell line.

The most striking result was from the pMybDelR1/2/3 construct. Although deletion of R1/2 and R3 individually increased c-myb promoter activity, the additional 15bp deletion of Region 3 from pMybDelR1/2

which results in complete removal of all the GGA repeats from the c-myb promoter, showed almost complete loss of luciferase activity in CCRF-CEM cells that ranges only 1~3% of the pMybWT construct. It is important to note that luciferase activity was still measurable (~50-fold over background) from the pMybDelR1/2/3 plasmids. Because of the striking loss of promoter activity by deletion of the GGA repeats in all cell lines tested, another pMybDelR1/2/3 construct was tested, and these results were confirmed with a second construct plasmid, Our results show no unintended artifact from the site-directed mutagenesis, and that at least one $(GGA)_4$ repeat region is required for c-myb promoter activity, and we speculate that duplication of the $(GGA)_4$ motif is used by the myb promoter to finely tune c-myb expression. Collectively, these data show that the c-myb GGA repeats are critical for regulating c-myb expression, and suggest that DNA secondary structures, specifically T:H:H:Ts formed by adjacent $(GGA)_4$ motifs, negatively regulate c-myb expression.

The transcription factor MAZ binds to the c-myb GGA repeat region.

Since our data demonstrated that the c-myb GGA repeat region is a critical *cis* element for controlling c-myb promoter activity, we investigated the possible protein:DNA interactions in the region. First, we searched for putative transcription factors for the c-myb GGA repeat region from +2 to +73 with the computer program MatInspector from GenoMatix (www.genomatix.de) (Supplementary Data). MAZ (Myc Associated Zinc finger), RXR, PU.1 and VDR were identified as transcription factors which have multiple potential binding sites in the GGA repeat region. We performed EMSAs to evaluate the binding of nuclear proteins from the leukemia cell line K562 to the myb GGA repeats in the presence of competitor oligos with the consensus binding sites for these transcription factors. Since the sequence of the GGA repeat region is repetitive we divided the GGA repeat region into three regions (VIII, IX, and X), each of which includes one of the $(GGA)_4$ repeat regions (R1, R2, and R3, respectively) for competition EMSAs (Figure 5B) and forms a stable duplex with a Tm > 64ºC. In the competition EMSA for investigating MAZ binding, we first incubated K562 cell nuclear extract with 0, 25X or 100X molar excess of a non-labeled double stranded competitor bearing a consensus MAZ binding site or mutant MAZ binding site and then the labeled double stranded probes ODN VIII, IX, and X, representing the three $(GGA)_4$ regions of the c-myb promoter, were added to the reaction. The competition EMSAs in Figure 5B demonstrate that the c-myb GGA duplex repeat region avidly interacts with multiple nuclear proteins, but several of the protein:DNA complexes were eliminated by the MAZ consensus competitor but not the MAZ mutant competitor. We also used consensus binding sequences for PU.1, RXR, and VDR in competition EMSAs.

These data are summarized in Figure 5C, and the other competition EMSAs are shown as Supplementary Data. These data provide preliminary evidence that MAZ, PU.1, RXR, and VDR have one or more binding sites in the c-myb GGA repeats.

To confirm MAZ binding to the c-myb promoter, we purified MAZ protein for EMSAs and DNase I footprinting studies. Figure 6A shows that MAZ binds to all three duplex probes representing the three GGA repeat regions of the c-myb promoter and two MAZ-probe complexes indicates that MAZ may bind to the duplex probes as a monomer or a dimer. DNase I digestion was performed on a 260bp fragment of the c-myb promoter in the presence of either a negative control FLGA peptides (Figure 6B, lane 2), or purified FLAG-MAZ (Figure 6B, lane 3) and only FLAG-MAZ showed protection from DNase I digestion spanning the GGA repeat region but not FLAG peptides, confirming our competition EMSA data that MAZ has multiple binding sites in the c-myb GGA repeats.

MAZ binds to the G-quadruplexes formed in the c-myb GGA repeat region and represses promoter activity

MAZ was reported to bind to the G-quadruplex formed by the G-rich region of the diabetes susceptibility locus *IDDM2* (24). In order to test if MAZ can also bind to the G-quadruplexes formed in the c-myb GGA repeat region we performed EMSAs with ODNs folded into T:H:H:T structures in the presence of FLAG-MAZ. ODN VI contains c-myb GGA repeat regions 1 and 2 and forms a stable T:H:H:T. ODN V (perfect $(GGA)_8$ sequence) and ODN VII (PPG substituted analog of ODN V) were studied as positive and negative controls, respectively. Figure 7 shows a MAZ protein:DNA interaction that is specific for the T:H:H:T structure. The band denoted as T:H:H:T-MAZ complex is observed only with ODNs V and VI, but not with ODN VII, the PPG substituted control, and only in the presence of MAZ. Nonspecific protein:DNA interactions, presumably form proteins in the reticulocyte lysate, are seen with all three ODNs in the presence or absence of MAZ. We investigated the role of MAZ in regulation of c-myb expression via cotransfection of pMybWT with the MAZ expression plasmid BRB112 in HEK 293 cells (Figure 8). Forced overexpression of MAZ dramatically decreased c-myb promoter activity, demonstrating that MAZ is a repressor of the c-myb promoter.

Collectively, this study presents the first evidence that the c-myb GGA repeat region forms T:H:H:T G-quadruplexes capable of binding to a transcriptional repressor protein. The GGA repeat region acts as a

repressor of c-myb expression by folding into a G-quadruplex structure and recruiting transcriptional repressors such as MAZ, while needed for basal transcription.

DISCUSSION

In support of previous NMR studies (19;20), our findings from DMS protection, CD, and polymerase arrest assays show that the GGA repeat region in the c-myb promoter is the first example of a genomic sequence that forms T:H:H:T G-quadruplex-like structures, involving two adjacent $(GGA)_4$ repeats. Several G-quadruplex regions have been identified in promoters and shown to be critical for regulation of promoter activity. The G-quadruplexes formed in the c-myc and Kras promoters inhibit promoter activity (26;27), whereas, the G-quadruplex formed in the human insulin gene activates the promoter (24;28), which suggest that G-quadruplex structure can alter gene expression. Our luciferase data showed that the c-myb G-quadruplex forming region is critical for regulation of c-myb expression, and the deletion studies demonstrated that the removal of one or two $(GGA)_4$ motifs in the c-myb promoter increases promoter activity, and is inversely correlated with the stability of the T:H:H:T G-quadruplexes formed by the remaining $(GGA)_4$ motifs in the mutant c-myb promoter. Interestingly, the GGA repeat region R1 and R3 that forms the most stable T:H:H:T in the c-myb promoter are absolutely conserved in the human and murine myb GGA repeat region, which implies that R1 and R3 are critical for regulation of myb expression. These findings indicate that the T:H:H:Ts formed in the c-myb promoter act as transcriptional repressors, and the c-myb promoter may regulate its activity by altering the stability of T:H:H:Ts via changing dimerization partners.

The mechanism by which G-quadruplexes can be transcriptional activators or inhibitors is not clear. There are many studies showing that promoter regions that support G-quadruplex formation are usually also binding sites for one or more important transcription factors (29-33). For instance, the c-myc NHE G-quadruplex forming region involves various DNA binding proteins, such as the double strand binding protein Sp1 (29), the single strand binding proteins CNBP (30), hnRNP K (31), and possibly yet to be discovered proteins that can recognize the G-quadruplex formed by this sequence. These protein interactions are probably in fine balance and competing for potentially complementary or exclusive binding sites. Interaction with transcription factors specific for a certain DNA conformation would shift the equilibrium to the DNA conformation at the region, which would be critical for determining promoter activity. Supporting this model of transcriptional regulation by

recognition of different DNA conformations, the study on the ErbB2 Hr-DNA formed in GGA repeats homologous to those in the c-myb promoter displaced the binding of the critical transcription factor Ets in the ErbB2 GGA repeat region, conversely, Ets binding inhibited Hr-DNA formation (34). This observation suggests that secondary DNA structures and protein:DNA interactions can influence each other to either activate or suppress gene expression. In agreement with this concept, our EMSA results demonstrate that the c-myb GGA repeat region actively interacts with many nuclear proteins (Figure 5) when it is double stranded, which might mean that T:H:H:T formation downregulates promoter activity by simply displacing double strand binding nuclear proteins.

The c-myb GGA repeat region is a hot spot for nuclear protein interaction as shown in Figure 5. Several previous studies showed that GGA repeats in a gene promoter were critical transcriptional activator binding sites (21;22). Similarly, our luciferase assay results demonstrated that the GGA repeat region is essential for c-myb promoter activity, which is abolished when the GGA repeats are deleted (Figure 4), probably due to the loss of transcription factor binding sites. These results indicate that the GGA repeat region has to be available for transcriptional activators to bind to the promoter and may imply that G-quadruplex formation, if stabilized by a small molecule, could also markedly inhibit c-myb expression making the region unavailable for transcription factor binding. The discovery and development of small molecule ligands for G-quadruplexes is the subject of investigation by several research groups (35;36).

We identified the Myc-associated Zinc finger protein (MAZ) as one of the transcription factors that bind to the GGA repeat region of c-myb, and showed that MAZ can bind to both ds DNA and T:H:H:T conformations of the c-myb promoter. MAZ was first identified as a GA box binding transcription factor in the c-myc promoter, and was shown to control P1 and P2 promoter activities of the c-myc gene (37-40). MAZ is constitutively expressed in various tissues (41) but abnormal MAZ expression is found in the terminal phase of chronic myelogenous leukemia (CML), suggesting that overexpression of MAZ plays a role in the progression of CML (42). The functional role of MAZ is dependent on the target gene and some promoters are activated by MAZ (43-45), while others are repressed (46;47). One possible explanation for the dual roles of MAZ may be that different cofactors interact with MAZ depending on the target promoter. Song *et al.* Showed that MAZ acts as a negative regulator via physical interaction with HDAC (Histone deacetylase complex) (47) and Jordarn-Scuitto *et al.* demonstrated that MAZ interacts with FAC1 (Fetal Alzheimer's clone 1), a truncation of the chromatin remodeler BPTF

(bromodomain and PHD domain transcription factor) (48;49). Interestingly, MAZ and the MAZ-like protein THZif-1 (triple-helix-binding zinc-finger protein -1) have now been shown to bind to secondary DNA structures (24;50). The G-quadruplex structure formed in the IDDM2 locus, a critical regulator of IDDM2 promoter activity, is recognized by MAZ, however, MAZ does not bind to the unfolded form of the G-quadruplex forming region (24). Moreover, the MAZ-like protein THZif-1 was shown to interact with the triplex formed in the c-myc NHE, which is both crucial for c-myc promoter activity, and more recently investigated by several groups as a G-quadruplex forming element (26;50-52). These observations suggest that regulation of promoter activity by MAZ and related proteins may involve recognition of secondary DNA structures. Some proteins, such as the Repressor-activator protein 1 RAP1, Thrombin, DNA topoisomerase I have been shown to stabilize G-quadruplexes (53-55). Other proteins, including RecQ helicases, RPA (Replication Protein A), and POT1 (Protection of Telomere 1), may inhibit secondary structure formation or serve to resolve secondary DNA structures (56-58). In the case of G-quadruplexes formed in gene promoters, we propose that these genes are regulated by mechanisms involving single stranded, double stranded, and folded DNA binding proteins that may inhibit or promote G-quadruplex formation. Furthermore, it is possible that some proteins can bind more than one DNA conformations, as we have shown with MAZ in this study (Figures 5 and 7). Since MAZ has dual binding activities both to double stranded and G-quadruplex DNA, regulation of promoter activity by MAZ might be determined by the cofactors that interact with MAZ. It is not known how MAZ recruits different cofactors, but it may depend on levels of MAZ in the nucleus or on posttranslational modifications, such as phosphorylation of MAZ. It is also possible that MAZ binding to double stranded or G-quadruplex DNA could result in changes in available transactivation domains of MAZ, which may allow different groups to interact with MAZ to determine the role of MAZ. Moreover, the c-myb GGA repeat region is the first identified G-quadruplex forming element in a transcribed region which indicates that T:H:H:T formation in the c-myb promoter may play a role as a transcriptional repressor not only by changing transcription factor interaction but also by acting as a roadblock of a transcriptional machinery. Forced overexpression of MAZ dramatically downregulates c-myb promoter activity (Figure 8), indicating that MAZ is a transcriptional repressor of the c-myb promoter. However, these data do not show whether repression of c-myb expression is due to MAZ binding to duplex DNA or the T:H:H:T structure.

The c-myb GGA repeat region is very complex, involving DNA conformational changes from double stranded to G-quadruplex forms which subsequently alter transcription factor binding to the region. In spite of this complexity, we first showed the importance of the T:H:H:T G-quadruplex forming region of the c-myb promoter in controlling promoter activity, identified MAZ as a T:H:H:T binding transcription factor, and presented preliminary evidence that MAZ is a repressor of the c-myb promoter. Further study is needed to discriminate DNA conformation dependent roles for MAZ in regulation of the c-myb promoter, to identify the cofactors which bind to MAZ depending on DNA structure, and to elucidate what determines MAZ binding to either G-quadruplex or double stranded DNA.

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Reference List

1. Oh,I.H. and Reddy,E.P. (1999) The myb gene family in cell growth, differentiation and apoptosis. *Oncogene*, **18**, 3017-3033.

2. Todokoro,K., Watson,R.J., Higo,H., Amanuma,H., Kuramochi,S., Yanagisawa,H. and Ikawa,Y. (1988) Down-regulation of c-myb gene expression is a prerequisite for erythropoietin-induced erythroid differentiation. *Proc.Natl.Acad.Sci.U.S.A*, **85**, 8900-8904.

3. Yi,H.K., Nam,S.Y., Kim,J.C., Kim,J.S., Lee,D.Y. and Hwang,P.H. (2002) Induction of apoptosis in K562 cells by dominant negative c-myb. *Exp.Hematol.*, **30**, 1139-1146.

4. Gonda,T.J. and Metcalf,D. (1984) Expression of myb, myc and fos proto-oncogenes during the differentiation of a murine myeloid leukaemia. *Nature*, **310**, 249-251.

5. Ramsay,R.G., Thompson,M.A., Hayman,J.A., Reid,G., Gonda,T.J. and Whitehead,R.H. (1992) Myb expression is higher in malignant human colonic carcinoma and premalignant adenomatous polyps than in normal mucosa. *Cell Growth Differ.*, **3**, 723-730.

6. Griffin,C.A. and Baylin,S.B. (1985) Expression of the c-myb oncogene in human small cell lung carcinoma. *Cancer Res.*, **45**, 272-275.

7. Slamon,D.J., Boone,T.C., Murdock,D.C., Keith,D.E., Press,M.F., Larson,R.A. and Souza,L.M. (1986) Studies of the human c-myb gene and its product in human acute leukemias. *Science*, **233**, 347-351.

8. Luger,S.M., O'Brien,S.G., Ratajczak,J., Ratajczak,M.Z., Mick,R., Stadtmauer,E.A., Nowell,P.C., Goldman,J.M. and Gewirtz,A.M. (2002) Oligodeoxynucleotide-mediated inhibition of c-myb gene expression in autografted bone marrow: a pilot study. *Blood*, **99**, 1150-1158.

9. Bender,T.P., Thompson,C.B. and Kuehl,W.M. (1987) Differential expression of c-myb mRNA in murine B lymphomas by a block to transcription elongation. *Science*, **237**, 1473-1476.

10. Watson,R.J. (1988) A transcriptional arrest mechanism involved in controlling constitutive levels of mouse c-myb mRNA. *Oncogene*, **2**, 267-272.

11. Watson,R.J. (1988) Expression of the c-myb and c-myc genes is regulated independently in differentiating mouse erythroleukemia cells by common processes of premature transcription arrest and increased mRNA turnover. *Mol.Cell Biol.*, **8**, 3938-3942.

12. Yuan,W. (2000) Intron 1 rather than 5' flanking sequence mediates cell type-specific expression of c-myb at level of transcription elongation. *Biochim.Biophys.Acta*, **1490**, 74-86.

13. Guerra,J., Withers,D.A. and Boxer,L.M. (1995) Myb binding sites mediate negative regulation of c-myb expression in T-cell lines. *Blood*, **86**, 1873-1880.

14. McCann,S., Sullivan,J., Guerra,J., Arcinas,M. and Boxer,L.M. (1995) Repression of the c-myb gene by WT1 protein in T and B cell lines. *J.Biol.Chem.*, **270**, 23785-23789.

15. Perrotti,D., Melotti,P., Skorski,T., Casella,I., Peschle,C. and Calabretta,B. (1995) Overexpression of the zinc finger protein MZF1 inhibits hematopoietic development from embryonic stem cells: correlation with negative regulation of CD34 and c-myb promoter activity. *Mol.Cell Biol.*, **15**, 6075-6087.

16. Bellon,T., Perrotti,D. and Calabretta,B. (1997) Granulocytic differentiation of normal hematopoietic precursor cells induced by transcription factor PU.1 correlates with negative regulation of the c-myb promoter. *Blood*, **90**, 1828-1839.

17. Sullivan,J., Feeley,B., Guerra,J. and Boxer,L.M. (1997) Identification of the major positive regulators of c-myb expression in hematopoietic cells of different lineages. *J.Biol.Chem.*, **272**, 1943-1949.

18. Nicolaides,N.C., Correa,I., Casadevall,C., Travali,S., Soprano,K.J. and Calabretta,B. (1992) The Jun family members, c-Jun and JunD, transactivate the human c-myb promoter via an Ap1-like element. *J.Biol.Chem.*, **267**, 19665-19672.

19. Matsugami,A., Ouhashi,K., Kanagawa,M., Liu,H., Kanagawa,S., Uesugi,S. and Katahira,M. (2001) An intramolecular quadruplex of (GGA)(4) triplet repeat DNA with a G:G:G:G tetrad and a G(:A):G(:A):G(:A):G heptad, and its dimeric interaction. *J.Mol.Biol.*, **313**, 255-269.

20. Matsugami,A., Okuizumi,T., Uesugi,S. and Katahira,M. (2003) Intramolecular higher order packing of parallel quadruplexes comprising a G:G:G:G tetrad and a G(:A):G(:A):G(:A):G heptad of GGA triplet repeat DNA. *J.Biol.Chem.*, **278**, 28147-28153.

21. Chamboredon,S., Briggs,J., Vial,E., Hurault,J., Galvagni,F., Oliviero,S., Bos,T. and Castellazzi,M. (2003) v-Jun downregulates the SPARC target gene by binding to the proximal promoter indirectly through Sp1/3. *Oncogene*, **22**, 4047-4061.

22. Hafner,M., Zimmermann,K., Pottgiesser,J., Krieg,T. and Nischt,R. (1995) A purine-rich sequence in the human BM-40 gene promoter region is a prerequisite for maximum transcription. *Matrix Biol.*, **14**, 733-741.

23. De Armond,R., Wood,S., Sun,D., Hurley,L.H. and Ebbinghaus,S.W. (2005) Evidence for the presence of a guanine quadruplex forming region within a polypurine tract of the hypoxia inducible factor 1alpha promoter. *Biochemistry*, **44**, 16341-16350.

24. Lew,A., Rutter,W.J. and Kennedy,G.C. (2000) Unusual DNA structure of the diabetes susceptibility locus IDDM2 and its effect on transcription by the insulin promoter factor Pur-1/MAZ. *Proc.Natl.Acad.Sci.U.S.A*, **97**, 12508-12512.

25. Keniry,M.A. (2000) Quadruplex structures in nucleic acids. *Biopolymers*, **56**, 123-146.

26. Siddiqui-Jain,A., Grand,C.L., Bearss,D.J. and Hurley,L.H. (2002) Direct evidence for a G-quadruplex in a promoter region and its targeting with a small molecule to repress c-MYC transcription. *Proc.Natl.Acad.Sci.U.S.A*, **99**, 11593-11598.

27. Cogoi,S. and Xodo,L.E. (2006) G-quadruplex formation within the promoter of the KRAS proto-oncogene and its effect on transcription. *Nucleic Acids Res.*, **34**, 2536-2549.

28. Hammond-Kosack,M.C., Dobrinski,B., Lurz,R., Docherty,K. and Kilpatrick,M.W. (1992) The human insulin gene linked polymorphic region exhibits an altered DNA structure. *Nucleic Acids Res.*, **20**, 231-236.

29. DesJardins,E. and Hay,N. (1993) Repeated CT elements bound by zinc finger proteins control the absolute and relative activities of the two principal human c-myc promoters. *Mol.Cell Biol.*, **13**, 5710-5724.

30. Michelotti,E.F., Tomonaga,T., Krutzsch,H. and Levens,D. (1995) Cellular nucleic acid binding protein regulates the CT element of the human c-myc protooncogene. *J.Biol.Chem.*, **270**, 9494-9499.

31. Takimoto,M., Tomonaga,T., Matunis,M., Avigan,M., Krutzsch,H., Dreyfuss,G. and Levens,D. (1993) Specific binding of heterogeneous ribonucleoprotein particle protein K to the human c-myc promoter, in vitro. *J.Biol.Chem.*, **268**, 18249-18258.

32. Ji,L., Mochon,E., Arcinas,M. and Boxer,L.M. (1996) CREB proteins function as positive regulators of the translocated bcl-2 allele in t(14;18) lymphomas. *J.Biol.Chem.*, **271**, 22687-22691.

33. Heckman,C., Mochon,E., Arcinas,M. and Boxer,L.M. (1997) The WT1 protein is a negative regulator of the normal bcl-2 allele in t(14;18) lymphomas. *J.Biol.Chem.*, **272**, 19609-19614.

34. Scott,G.K., Chang,C.H., Erny,K.M., Xu,F., Fredericks,W.J., Rauscher,F.J., III, Thor,A.D. and Benz,C.C. (2000) Ets regulation of the erbB2 promoter. *Oncogene*, **19**, 6490-6502.

35. Hurley,L.H., Wheelhouse,R.T., Sun,D., Kerwin,S.M., Salazar,M., Fedoroff,O.Y., Han,F.X., Han,H., Izbicka,E. and Von Hoff,D.D. (2000) G-quadruplexes as targets for drug design. *Pharmacol.Ther.*, **85**, 141-158.

36. Kerwin,S.M. (2000) G-Quadruplex DNA as a target for drug design. *Curr.Pharm.Des*, **6**, 441-478.

37. Pyrc,J.J., Moberg,K.H. and Hall,D.J. (1992) Isolation of a novel cDNA encoding a zinc-finger protein that binds to two sites within the c-myc promoter. *Biochemistry*, **31**, 4102-4110.

38. Komatsu,M., Li,H.O., Tsutsui,H., Itakura,K., Matsumura,M. and Yokoyama,K.K. (1997) MAZ, a Myc-associated zinc finger protein, is essential for the ME1a1-mediated expression of the c-myc gene during neuroectodermal differentiation of P19 cells. *Oncogene*, **15**, 1123-1131.

39. Bossone,S.A., Asselin,C., Patel,A.J. and Marcu,K.B. (1992) MAZ, a zinc finger protein, binds to c-MYC and C2 gene sequences regulating transcriptional initiation and termination. *Proc.Natl.Acad.Sci.U.S.A*, **89**, 7452-7456.

40. Izzo,M.W., Strachan,G.D., Stubbs,M.C. and Hall,D.J. (1999) Transcriptional repression from the c-myc P2 promoter by the zinc finger protein ZF87/MAZ. *J.Biol.Chem.*, **274**, 19498-19506.

41. Song,J., Murakami,H., Tsutsui,H., Tang,X., Matsumura,M., Itakura,K., Kanazawa,I., Sun,K. and Yokoyama,K.K. (1998) Genomic organization and expression of a human gene for Myc-associated zinc finger protein (MAZ). *J.Biol.Chem.*, **273**, 20603-20614.

42. Daheron,L., Salmeron,S., Patri,S., Brizard,A., Guilhot,F., Chomel,J.C. and Kitzis,A. (1998) Identification of several genes differentially expressed during progression of chronic myelogenous leukemia. *Leukemia*, **12**, 326-332.

43. Parks,C.L. and Shenk,T. (1996) The serotonin 1a receptor gene contains a TATA-less promoter that responds to MAZ and Sp1. *J.Biol.Chem.*, **271**, 4417-4430.

44. Parks,C.L. and Shenk,T. (1997) Activation of the adenovirus major late promoter by transcription factors MAZ and Sp1. *J.Virol.*, **71**, 9600-9607.

45. Williams,L.J. and Abou-Samra,A.B. (2000) The transcription factors SP1 and MAZ regulate expression of the parathyroid hormone/parathyroid hormone-related peptide receptor gene. *J.Mol.Endocrinol.*, **25**, 309-319.

46. Karantzoulis-Fegaras,F., Antoniou,H., Lai,S.L., Kulkarni,G., D'Abreo,C., Wong,G.K., Miller,T.L., Chan,Y., Atkins,J., Wang,Y. *et al.* (1999) Characterization of the human endothelial nitric-oxide synthase promoter. *J.Biol.Chem.*, **274**, 3076-3093.

47. Song,J., Ugai,H., Kanazawa,I., Sun,K. and Yokoyama,K.K. (2001) Independent repression of a GC-rich housekeeping gene by Sp1 and MAZ involves the same cis-elements. *J.Biol.Chem.*, **276**, 19897-19904.

48. Jordan-Sciutto,K.L., Dragich,J.M., Caltagarone,J., Hall,D.J. and Bowser,R. (2000) Fetal Alz-50 clone 1 (FAC1) protein interacts with the Myc-associated zinc finger protein (ZF87/MAZ) and alters its transcriptional activity. *Biochemistry*, **39**, 3206-3215.

49. Barak,O., Lazzaro,M.A., Lane,W.S., Speicher,D.W., Picketts,D.J. and Shiekhattar,R. (2003) Isolation of human NURF: a regulator of Engrailed gene expression. *EMBO J.*, **22**, 6089-6100.

50. Sakatsume,O., Tsutsui,H., Wang,Y., Gao,H., Tang,X., Yamauchi,T., Murata,T., Itakura,K. and Yokoyama,K.K. (1996) Binding of THZif-1, a MAZ-like zinc finger protein to the nuclease-hypersensitive element in the promoter region of the c-MYC protooncogene. *J.Biol.Chem.*, **271**, 31322-31333.

51. Freyer,M.W., Buscaglia,R., Kaplan,K., Cashman,D., Hurley,L.H. and Lewis,E.A. (2007) Biophysical studies of the c-MYC NHE III1 promoter: model quadruplex interactions with a cationic porphyrin. *Biophys.J.*, **92**, 2007-2015.

52. Yang,D. and Hurley,L.H. (2006) Structure of the biologically relevant G-quadruplex in the c-MYC promoter. *Nucleosides Nucleotides Nucleic Acids*, **25**, 951-968.

53. Giraldo,R., Suzuki,M., Chapman,L. and Rhodes,D. (1994) Promotion of parallel DNA quadruplexes by a yeast telomere binding protein: a circular dichroism study. *Proc.Natl.Acad.Sci.U.S.A*, **91**, 7658-7662.

54. Baldrich,E. and O'Sullivan,C.K. (2005) Ability of thrombin to act as molecular chaperone, inducing formation of quadruplex structure of thrombin-binding aptamer. *Anal.Biochem.*, **341**, 194-197.

55. Arimondo,P.B., Riou,J.F., Mergny,J.L., Tazi,J., Sun,J.S., Garestier,T. and Helene,C. (2000) Interaction of human DNA topoisomerase I with G-quartet structures. *Nucleic Acids Res.*, **28**, 4832-4838.

56. Mohaghegh,P., Karow,J.K., Brosh,J.R., Jr., Bohr,V.A. and Hickson,I.D. (2001) The Bloom's and Werner's syndrome proteins are DNA structure-specific helicases. *Nucleic Acids Res.*, **29**, 2843-2849.

57. Salas,T.R., Petruseva,I., Lavrik,O., Bourdoncle,A., Mergny,J.L., Favre,A. and Saintome,C. (2006) Human replication protein A unfolds telomeric G-quadruplexes. *Nucleic Acids Res.*, **34**, 4857-4865.

58. Zaug,A.J., Podell,E.R. and Cech,T.R. (2005) Human POT1 disrupts telomeric G-quadruplexes allowing telomerase extension in vitro. *Proc.Natl.Acad.Sci.U.S.A*, **102**, 10864-10869.

Table 1. Summary of oligonucleotides (ODNs) used in this study

		DMS protection	CD spectra	EMSA	DNA pol arrest	RNA pol arrest
c-myb GGA repeat region	wildtype	I			XI	XXI
	R1 mut	II			XII	
	R2 mut	III			XIII	
	R3 mut	IV			XIV	
	$(GGA)_8$ control		V	V	XVII	
	R1 and 2		VI	VI		
	PPG R1 and 2		VII	VII		
	R1 duplex			VIII		
	R2 duplex			IX		
	R3 duplex			X		
	R2 and 3				XV	XXV
	R3				XVI	XXVI

For detail sequences, see Supplementary Data.

Figure legends

Figure 1. The c-myb promoter and key transcription factors that regulate c-myb expression and T:H:H:T G-quadruplexes formed by $(GGA)_4$ or $(GGA)_8$

A. Transcription initiation site (+1) is indicated with an arrow. Inhibitors: c-Myb (-596 ~ -618), WT1 (-390 ~ -428), myeloid zinc finger 1(MZF-1, -50 ~ -64 and –162 ~ -178), and PU.1 binding site (+15 ~ +20). Activators: Ets (-251 ~ -270) and c-Jun /JunD (-138 ~ -162). Putative transcription factors that bind to the GGA repeat region include MAZ, RXR and VDR (italics), which have multiple binding sites in the GGA repeat region. The GGA repeat sequences in the murine c-myb promoter and the corresponding region in human c-myb are shown with the $(GGA)_4$ motifs are underlined. R2 and R3 have perfect $(GGA)_4$ sequences, and R1 has a nearly perfect sequence $(GGA)_3$(GGT). B. Left: $(GGA)_4$ forms a T:H and two T:Hs intramolecularlly dimerize to form a T:H:H:T G-quadruplex. Right: $(GGA)_8$ forms two T:Hs and those dimerize intramolecularlly, resulting in a T:H:H:T G-quadruplex. (Figure 1B Left and Right are from reference 19 and 20 and permission for using these figures is pending.)

Figure 2. Dimethylsulfate (DMS) protection assay and Circular Dichroism spectra

A. DMS methylation patterns are indicated by ○ (methylated), ◐ (partially mathylated), ● (unmethylated). The wild-type GGA repeat sequence (ODN I) showed protection from DMS methylation in R1 and R2 in the presence of potassium. The $(GGA)_4$ to $(GCA)_4$ mutants (ODNs II, III, and IV) showed protection in the two intact $(GGA)_4$ repeats in the presence of potassium. B. Circular dichroism spectra of ODN V, VI, and VII in the presence of KCl. ODNs V and VI showed CD signature of G-quadruplex but not ODN VII. Note that Ps in ODN VII means PPGs. C. Summary of the stability of the T:H:H:T G-quadruplexes formed in ODNs II, III, and IV.

Figure 3. RNA and DNA polymerase arrest assays

A. The wild-type and regional mutants of the c-myb GGA repeat region showed potassium dependent Taq polymerase arrest sites. The wild-type GGA repeat sequence (ODN XI) caused two arrest sites before R3 and R2 and the regional mutants showed a single arrest site before the first intact $(GGA)_4$. These bands were quantified by densitometry, and the percentage of arrest products is shown on the right panel of A. Note that the regions mutated from $(GGA)_4$ to $(GCA)_4$ are indicated as black ovals. B and C. DNA (B) and RNA (C) polymerase arrest assays with the regional deletion mutants of the c-myb GGA repeat region. Deletion of R1 (ODNs XV and XXV) resulted in a single arrest site at S1. Deletion of R1 and R2 (ODNs XVI and XXVI) arrests RNA polymerase at S1 but not DNA polymerase. The location of the arrest sites (S1 and S2) are shown schematically relative to GGA repeat regions (R1, R2, R3), the primer binding site (P), and the full-length extension product (FL) in the diagram adjacent to the autoradiogram.

Figure 4. Luciferase activity driven by the wild-type and GGA deleted c-myb promoters

The wild-type and regional deletion mutant c-myb promoter driven luciferase reporter constructs were transiently transected into the human leukemia cell line CCRF-CEM. Deletion of R1, R2, R3, or both R1 and R2 from the c-myb promoter increased luciferase activity in CCRT-CEM cells. The R1, R2, and R3 deletion mutant pMybDelR1/2/3 markedly reduced luciferase activity. * P value < 0.01.

Figure 5. Competition electrophoretic mobility shift assays (EMSA) with K562 nuclear extract

A. The DNA probes used for EMSAs are illustrated under the corresponding GGA repeat sequence. The probes are double stranded, but only the G-rich sequence is shown. B. The results from competition EMSAs using a MAZ competitor are shown. The protein:DNA complexes completely or almost completely eliminated by the MAZ competitor are indicated with arrows. C. Summary of competition EMSA results. The putative transcription factors shown to interact or not to interact with the c-myb GGA repeat region in competition EMSAs are listed.

Figure 6. Electrophoretic mobility shift and DNase I protection assays with FLAG-MAZ

A. EMSA. The complexes of FLAG-MAZ and the c-myb probes are indicated with arrows. B. DNase I protection assay. Lane 1: sequencing for guanines, lane 2: FLAG peptide, and lane 3: FLAG-MAZ. The area protected area from DNase I digestion is marked with a solid line.

Figure 7. MAZ binding to the G-quadruplexes formed by GGA repeat DNA

EMSA with ODNs V, VI, and VII in the presence or absence of TnT expressed MAZ. TnT - MAZ binds to the T:H:H:T G-quadruplexes formed in ODNs V and VI but does not to the ODN VII that cannot form a G-quadruplex. DNA-protein complexes formed with TnT-MAZ or nonspecific TnT-lysate protein and the probes are indicated with arrows.

Figure 8. Cotransfection of pMybWT with the MAZ expression plasmid BRB112

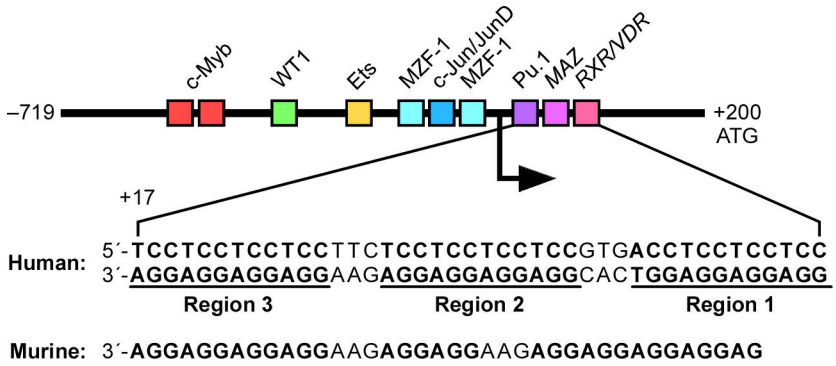
1μg of pMybWT and 0 to 3 μg of BRB112 were transfected into HEK 293 cells and luciferase activities were measured 24hrs after transfection. MAZ overexpression resulted in inhibition of c-myb promoter activity.

Figure 9. Working model for regulation of c-myb promoter activity via G-quadruplex formation in the c-myb GGA repeat region

A: The equilibrium between the duplex form, open, and G-quadruplex structure at the c-myb GGA repeat region. In the duplex form, transcriptional repressors and activators bind to the c-myb GGA repeat region in order to regulate c-myb expression levels and MAZ possibly act as a repressor of c-myb promoter by binding this form. In the G-quadruplex structure, duplex binding proteins are dissociated from the GGA repeat region and G-quadruplex binding proteins regulate G-quadruplex stability. MAZ may downregulate c-myb promoter activity via G-quadruplex binding at the GGA repeat region. B. Schematic representation of G-rich strand of the GGA repeat region. When locally unwounded, the c-myb GGA repeat region could form T:H:H:T G-quadruplexes with any two of three $(GGA)_4$ repeats in the region.

Figures

A.



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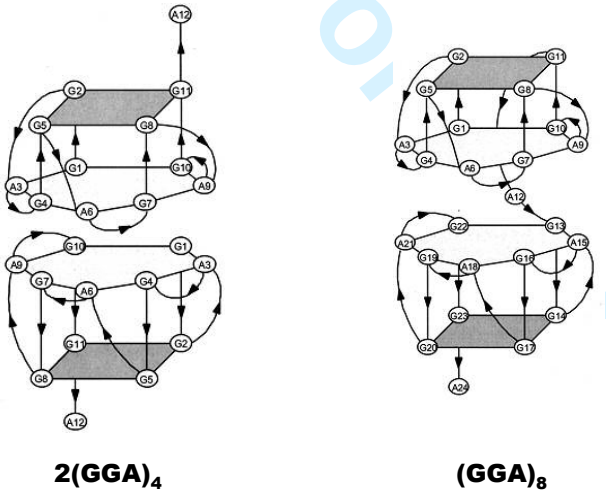


Figure 1. The c-myb promoter and key transcription factors that regulate c-myb expression and T:H:H:T G-quadruplexes formed by $(GGA)_4$ or $(GGA)_8$ from reference 19 and 20, respectively. (Permission for using these Figure 1B is pending.)

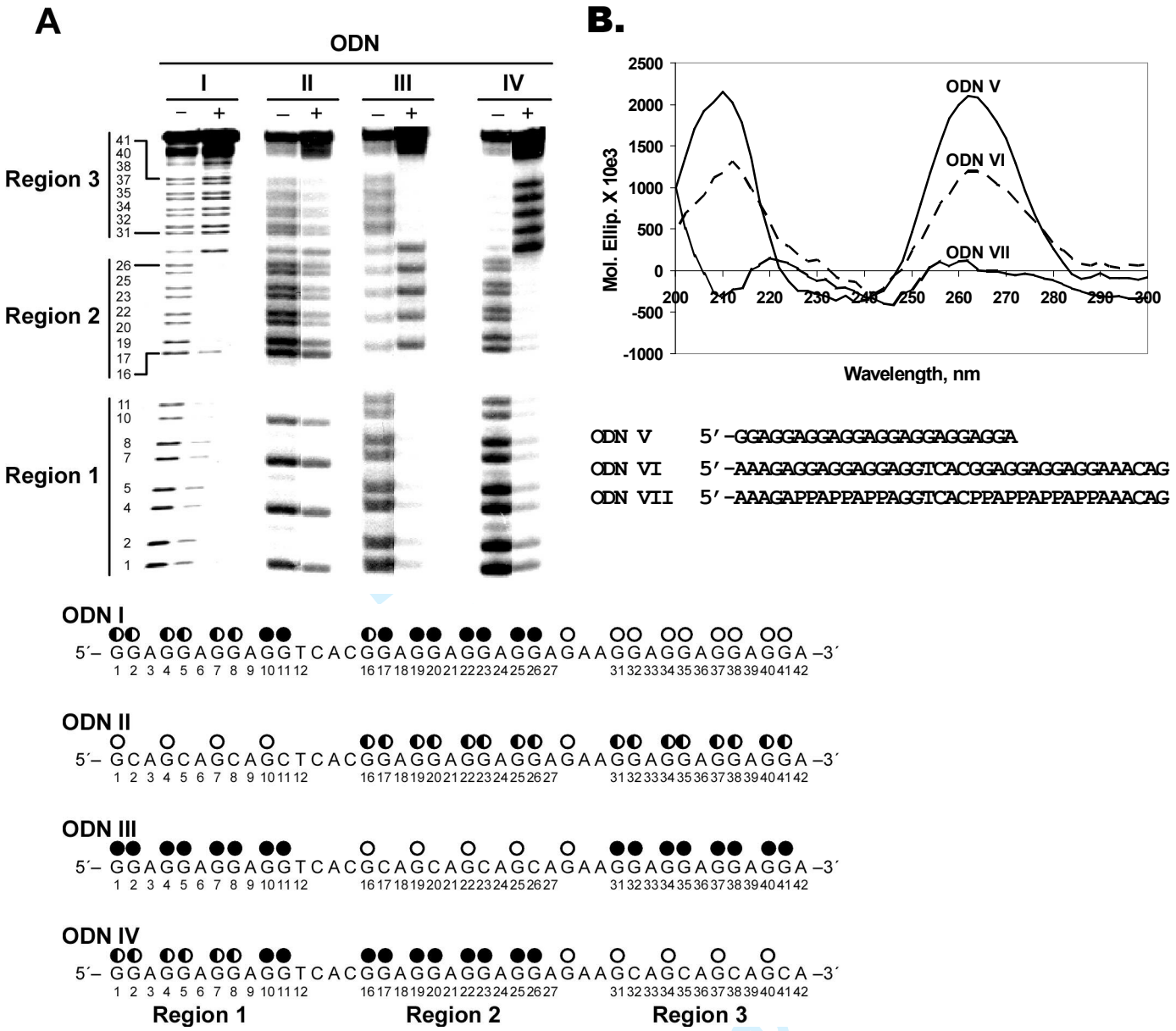


Figure 2. Dimethylsulfate (DMS) protection assay and Circular Dichroism spectra

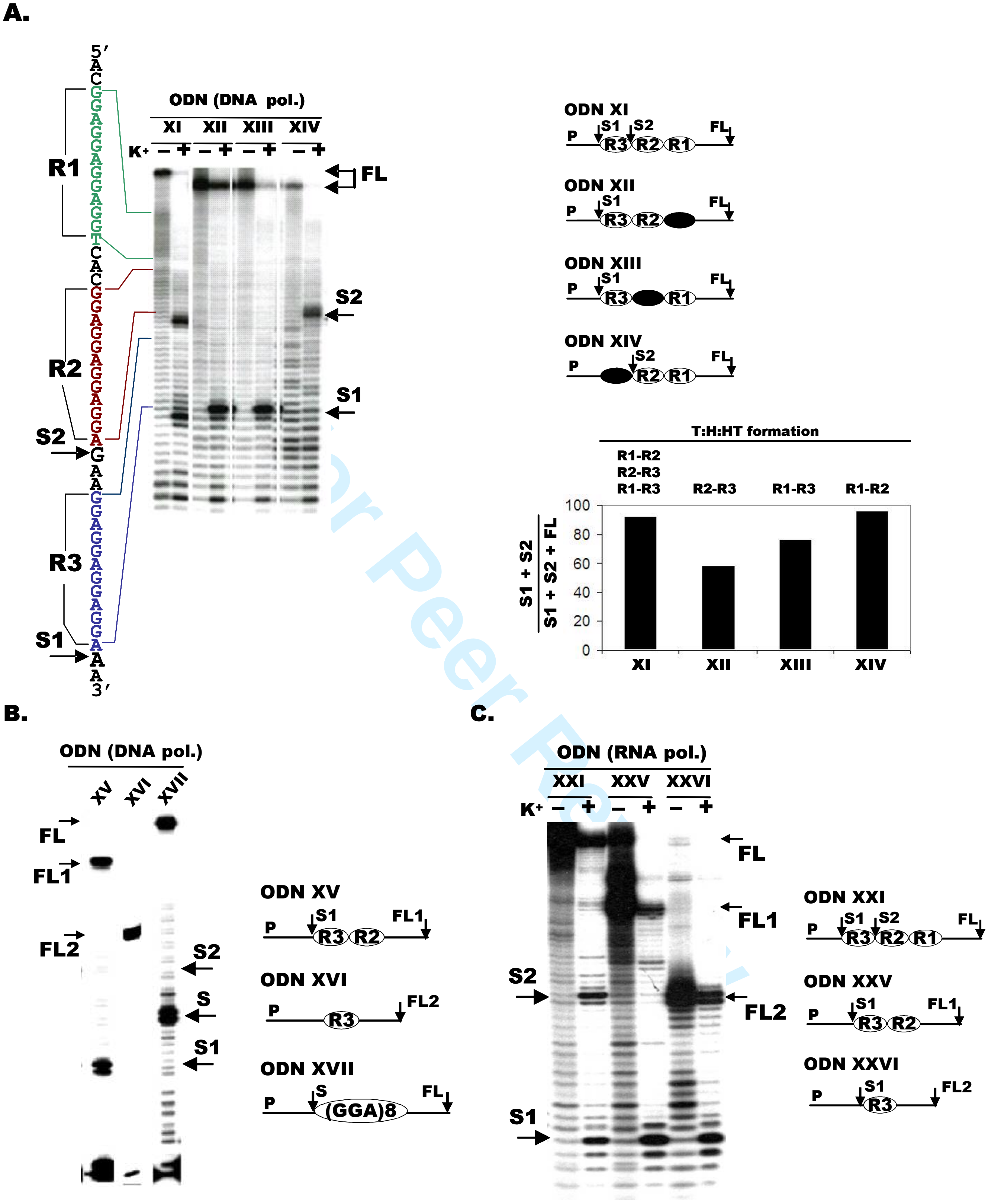


Figure 3. RNA and DNA polymerase arrest assays

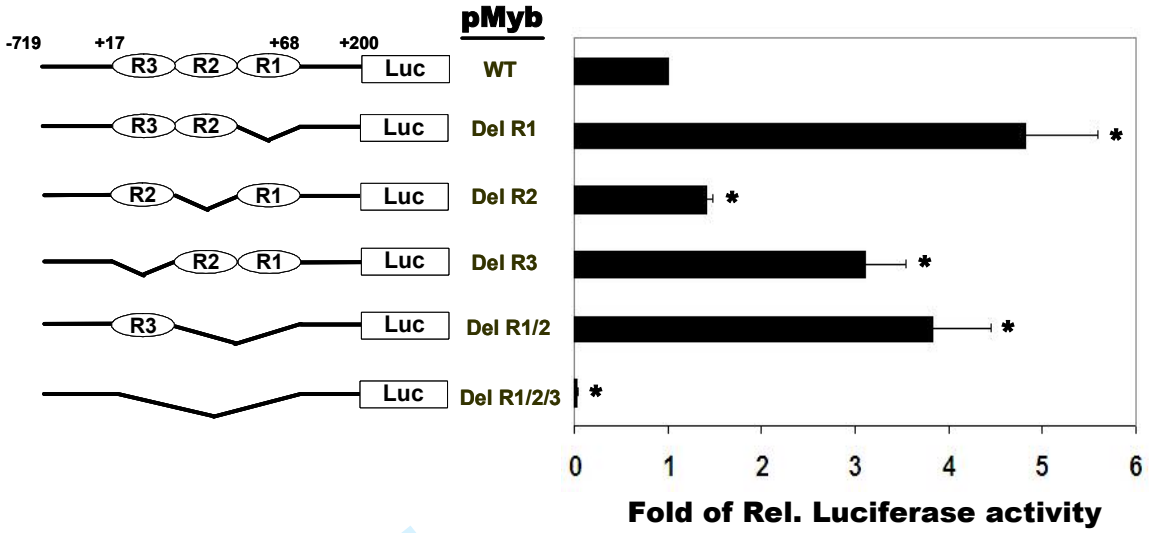
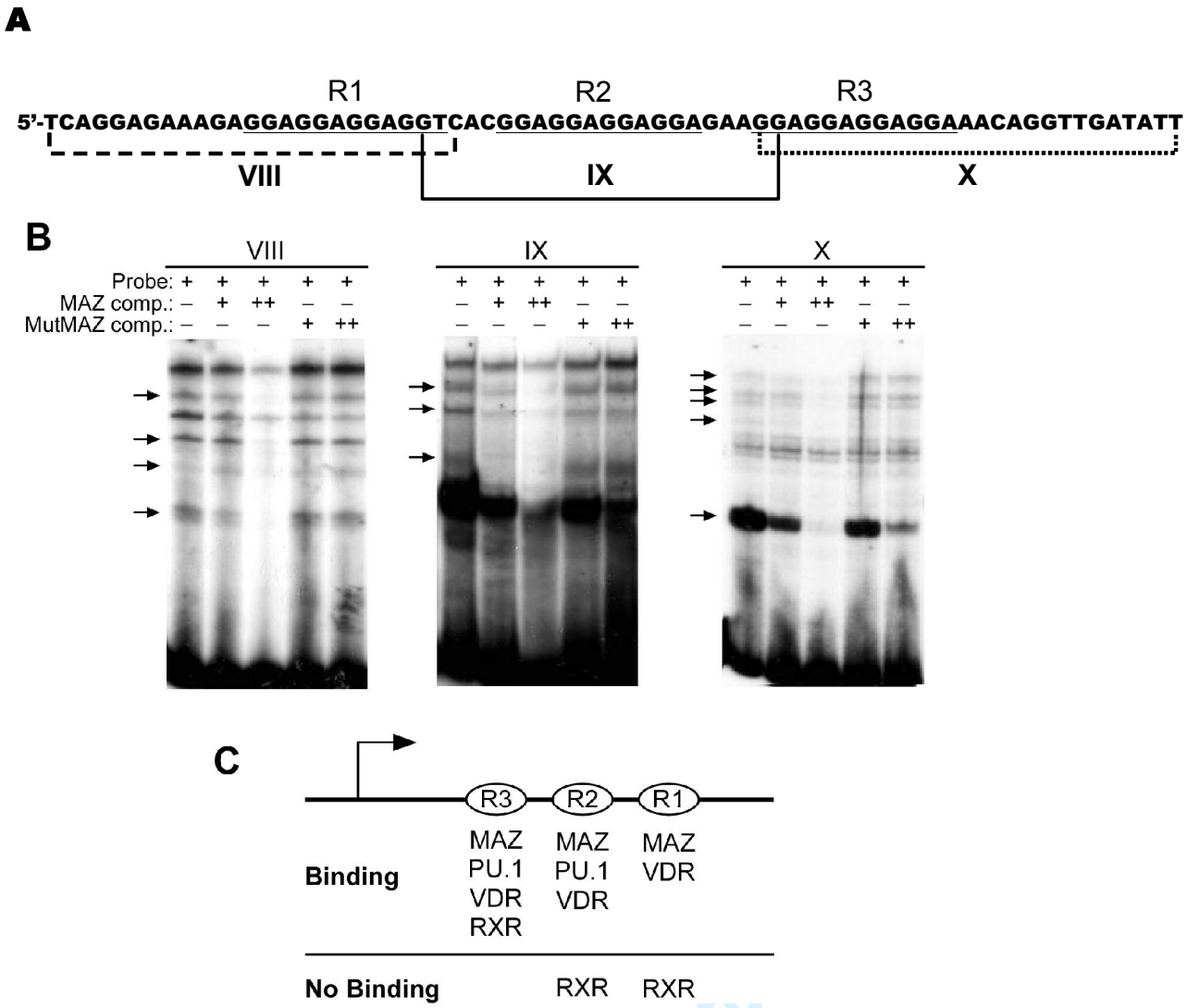


Figure 4. Luciferase activity driven by the wild-type and GGA deleted c-myb promoters



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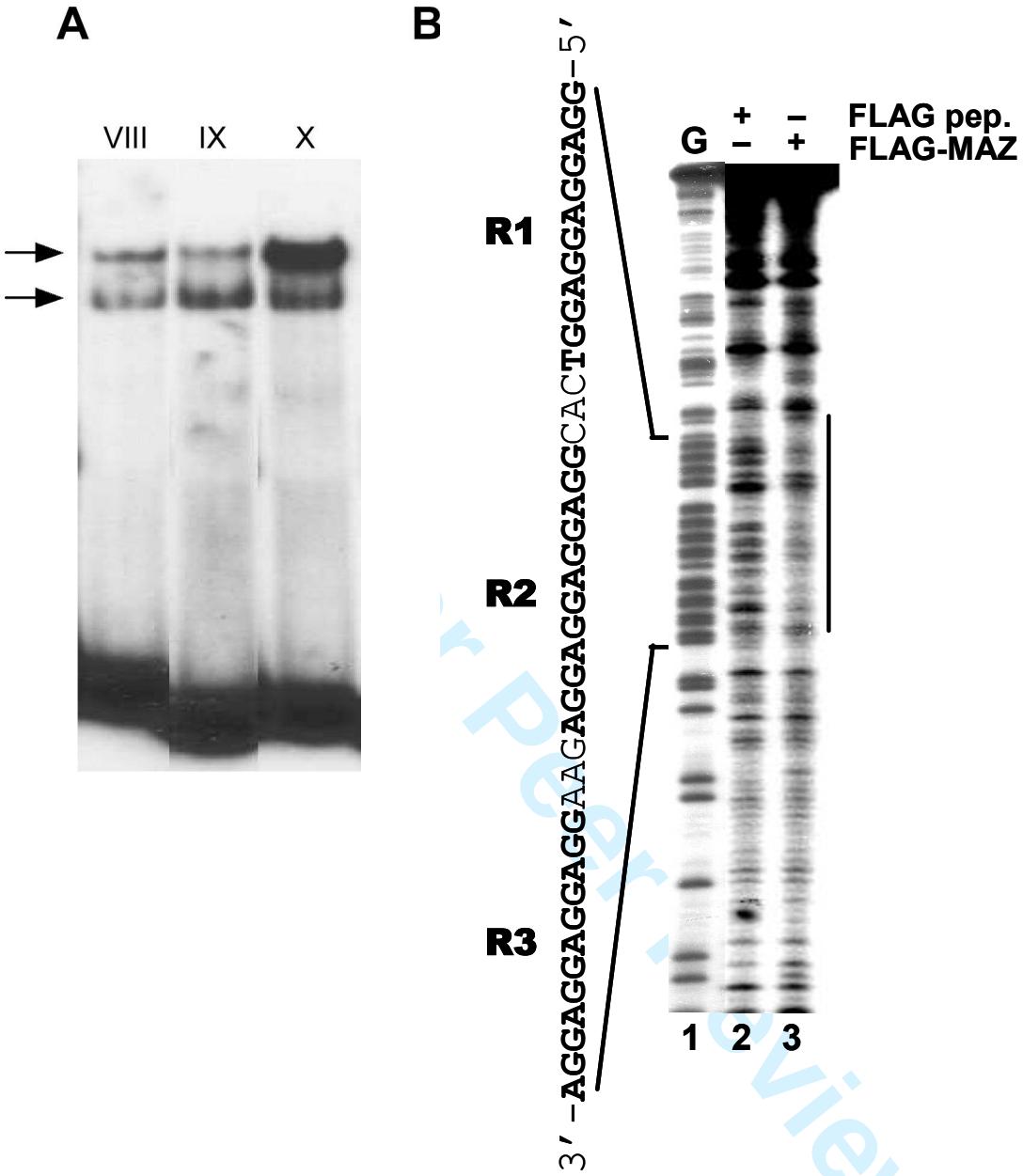
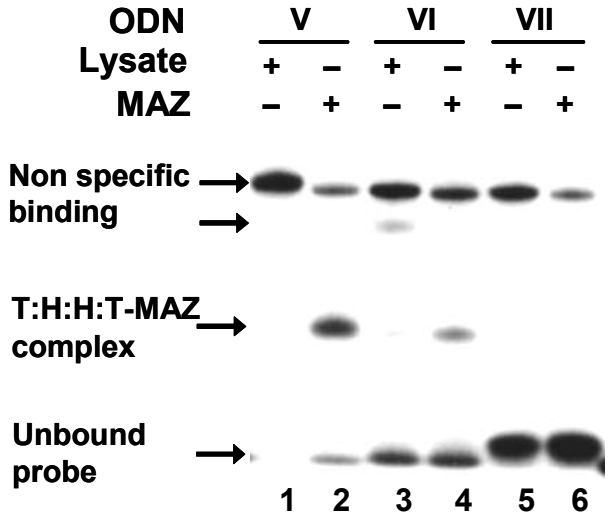


Figure 6. Electrophoretic mobility shift and DNase I protection assays with FLAG-MAZ



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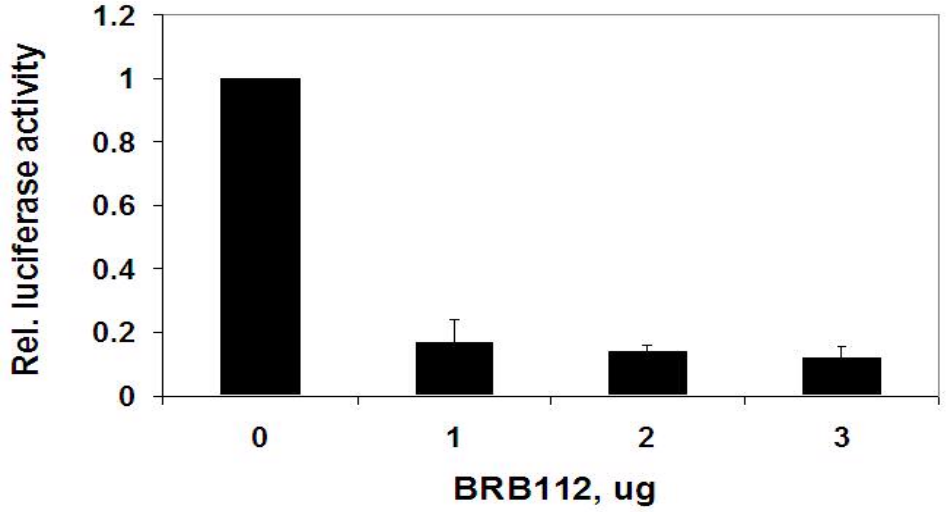
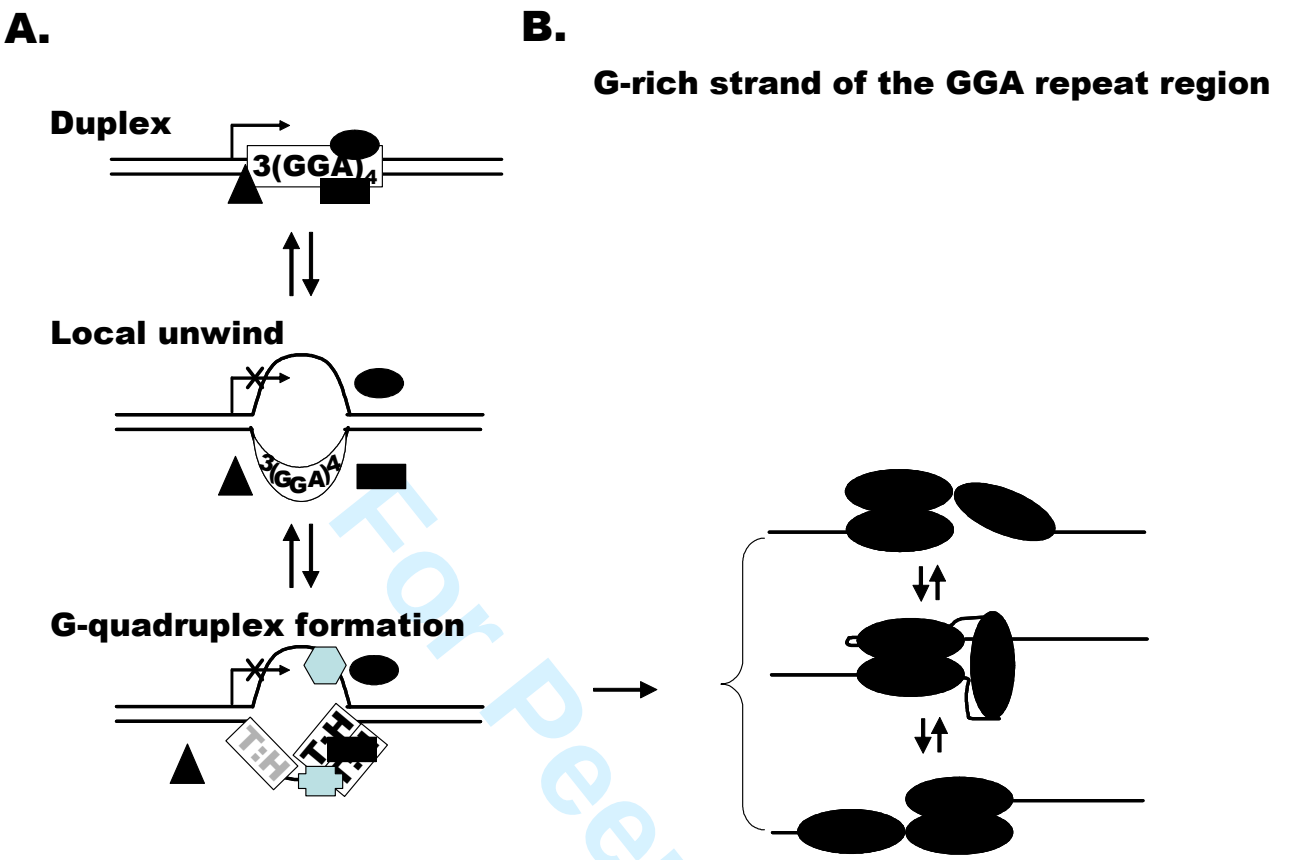


Figure 8. Cotransfection of pMybWT with BRB112 into HEK 293 cells



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